RayBio®Phospho-EGFR (Ser1070) and Pan EGFR ELISA Kit

For Measuring Phosph-EGFR (Ser 1070) and Pan EGFR in Human Cell Lysates

User Manual
(Revised Mar 15, 2012)

RayBio®Phospho-EGFR (Ser 1070) and Pan EGFR ELISA Kit Protocol

(Cat#: PEL-EGFR-S1070-T)

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I. INTRODUCTION

RayBio® Human Phospho-EGFR (Ser 1070) and Pan EGFR ELISA (Enzyme-Linked Immunosorbent Assay) kit is a very rapid, convenient and sensitive assay kit that can monitor the activation or function of important biological pathways in cell lysates. By determining phosphorylated EGFR protein in your experimental model system, you can verify pathway activation in your cell lysates. You can simultaneously measure numerous different cell lysates without spending excess time and effort in performing a Western Blot analysis.

This Sandwich ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of phospho-EGFR (Ser 1070) and pan EGFR (help normalize the results of phospho-EGFR from different cell lysate being compared) in human cell lysate. An anti-EGFR antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and phosphorylated and unphosphorylated EGFR present in a sample is bound to the wells by the immobilized antibody. The wells are washed and anti-phosphorylated EGFR (Ser1070) or biotinylated anti-pan-EGFR antibody is used to detect phosphorylated or pan EGFR. After washing away unbound antibody, HRP-conjugated anti-Rabbit IgG or HRP-Streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of EGFR (Ser1070) or pan EGFR bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

II. MATERIAL PROVIDED

1. EGFR Microplate (Item A): 96 wells (12 strips x 8 wells) coated with monoclonal anti-human EGFR.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Anti-phospho-EGFR (Ser 1070) (Item C): 1vial rabbit anti-human EGFR (Ser 1070).
4. HRP-conjugated Anti-rabbit IgG (Item D-1), 25 μl of 1000x concentrated HRP-conjugated anti-rabbit IgG.
5. Biotinylated-Anti-EGFR (Item L): 1 vial goat biotinylated anti-human EGFR.
6. HRP-Streptavidin concentrate (Item G): 200 μl of 600 fold concentrated HRP-Streptavidin concentrate.
7. Assay Diluent (Item E2): 15 ml of 5x concentrated buffer. For diluting cell lysate, Biotinylated antibody (Item L), HRP-conjugated anti-rabbit IgG (Item D-1) and HRP-Streptavidin (Item G) diluent.
8. TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
9. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.
10. Cell Lysate Buffer (Item J): 5 ml 2x Cell Lysate Buffer (not including protease and phosphatase inhibitors).
11. Positive Control A431S001-1 (Item K): 1 vial of lyophilized powder from A431 cell lysate.

III. STORAGE

Upon receipt, the kit should be stored at –20 °C. Please use within 6 months from the date of shipment. After initial use, Wash Buffer Concentrate (Item B), HRP-conjugated Anti-rabbit IgG (Item D-1), Assay Diluent (Item E2), HRP-Streptavidin (Item G), TMB One-Step Substrate Reagent (Item H), Stop Solution (Item I) and Cell Lysate Buffer (Item J) should be stored at 4 °C to avoid repeated freeze-thaw cycles. Return unused wells to the pouch containing desiccant pack, reseal along entire edge and store at –20 °C. Reconstituted Positive Control (Item K) should be stored at –70 °C.

IV. ADDITIONAL MATERIALS REQUIRED

1 Microplate reader capable of measuring absorbance at 450 nm.
2 Protease and Phosphatase inhibitors.
3 Shaker.
4 Precision pipettes to deliver 2 μl to 1 ml volumes.
5 Adjustable 1-25 ml pipettes for reagent preparation.
6 100 ml and 1 liter graduated cylinders.
7 Distilled or deionized water.
8 Tubes to prepare sample dilutions.

V. SAMPLE PREPARATION

Cell lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding the Cell Lysate Buffer. Solubilize cells at 4 x 10^7 cells/ml in 1x Cell Lysate Buffer (we recommend adding protease and phosphatase inhibitors to Cell Lysate Buffer prior to sample preparation). Pipette up and down to resuspend and incubate the lysates with shaking at 2 - 8° C for 30 minutes. Microcentrifuge at 13,000 rpm for 10 minutes at 2 - 8° C, and transfer the supernates into a clean test tube. Lysates should be used immediately or aliquoted and stored at –70 °C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use.

For the initial experiment, we recommend to do a serial dilution testing such as 5-fold and 100-fold dilution for your cell lysates with Assay Diluent (Item E2) before use.

Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empiricallys. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

Cell Lysate Buffer should be diluted 2-fold with deionized or distilled water before use (recommend to add protease and phosphatase inhibitors).

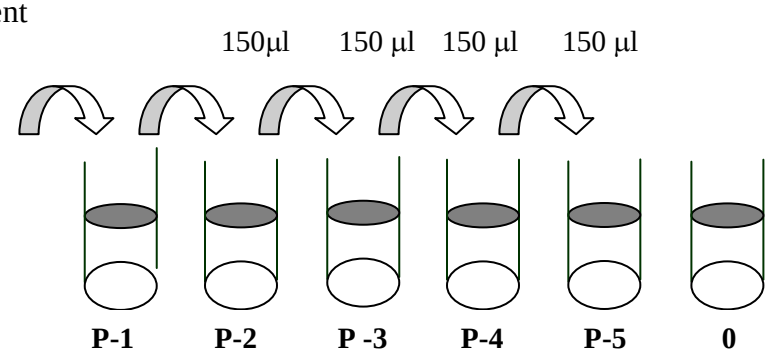
VI. REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.

2. Item E2, Assay Diluent should be diluted 5-fold with deionized or distilled water before use.

3. Briefly spin the Positive Control vial of Item K. Add 800 µl 1x Assay Diluent (Item E2, Assay Diluent should be diluted 5-fold with deionized or distilled water before use) into Item K vial to prepare a Positive Control (P-1) Solution (See i. Positive control of part IX. TYPICAL DATA for a typical result). Dissolve the powder thoroughly by a gentle mix. Pipette 300 µl 1x Assay Diluent into each tube. Use the Positive Control (1) to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. 1x Assay Diluent serves as the background.

Positive Control, Item K vial
+ 800 µl 1x Assay Diluent



4. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute

20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1x Wash Buffer.

5. Briefly spin the anti-phospho-EGFR (Ser 1070) (Item C) before use. Add 100 μl of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days. It can be used within one month If store at -80°C. Avoid repeated freeze-thaw cycles). The detection antibody concentrate should further be diluted 60-folds with 1x Assay Diluent and used in step 4 of Part VII Assay Procedure.

6. Briefly spin the HRP-conjugated anti-rabbit IgG (Item D-1), before use. Pipette up and down to mix gently. HRP-conjugated anti-rabbit IgG concentrate should be diluted 1,000-folds with 1x Assay Diuent.

7. Briefly spin the Detection Antibody vial (Item L) before use. Add 100 μl of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days. It can be used within one month If store at -80°C. Avoid repeated freeze-thaw cycles). The detection antibody concentrate should be diluted 200-folds with 1x Assay Diluent and used in step 4 of Part VI Assay Procedure.

8. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use since precipitation may form during storage. HRP-Streptavidin

concentrate should be diluted 600-fold with 1x Assay Diluent.

For example: Briefly spin the vial (Item G) and pipette up and down to mix gently . A*dd 20 μl of HRP-Streptavidin concentrate into a tube with 12 ml 1x Assay Diluent B to prepare a 600-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix well.*

9. Cell Lysate Buffer should be diluted 2-folds with deionized or distilled water before use (recommend to add protease and phosphatase inhibitors).

VII. ASSAY PROCEDURE:

1. Bring all reagents to room temperature (18 - 25°C) before use. It is recommended that all samples or Positive Control should be run at least in duplicate.

2. Add 100 μl of each sample or positive control into appropriate wells. Cover well with plate holder and incubate for 2.5 hours at room temperature or over night at 4°C with shaking.

3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 μl) using a multi-channel pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or

decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 μl of 1x anti-phospho-EGFR (Ser 1070) to corresponding well (your sample and positive control) for detecting phospho-EGFR (Ser1070) or 100 μl of 200 fold diluted biotinylated anti-EGFR to corresponding well (your sample, help normalize the results of phospho-EGFR from different cell lysate being compared) for detecting a pan EGFR. Incubate for 1.5 hour at room temperature with shaking.

5. Discar Discard the solution. Repeat the wash as in step 3.

6. Add 100 μl of 1x HRP-conjugated anti-rabbit IgG (see Reagent Preparation step 6) to detect rabbit anti-phospho-EGFR (Ser 1070) (corresponding well added rabbit anti-phospho-EGFR) or 100 μl of 600 fold diluted HRP-Streptavidin to detect biotinylatded anti-EGFR antibody (corresponding well added HRP-Streptavidin). Incubate for 1 hour at room temperature with shaking.

7. Discard the solution. Repeat the wash as in step 3.

8. Add 100 μl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with shaking.

9. Add 50 μl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.

⇩

2. Add 100 µl sample or positive control to each well.
   Incubate 2.5 hours at room temperature or over night at 4°C.

⇩

3. Add 100 µl prepared primary antibody to each well.
   Incubate 1.5 hour at room temperature.

⇩

4. Add 100 µl prepared secondary antibody solution.
   Incubate 1 hour at room temperature.

⇩

5. Add 100 µl TMB One-Step Substrate Reagent to each well.
   Incubate 30 minutes at room temperature.

⇩

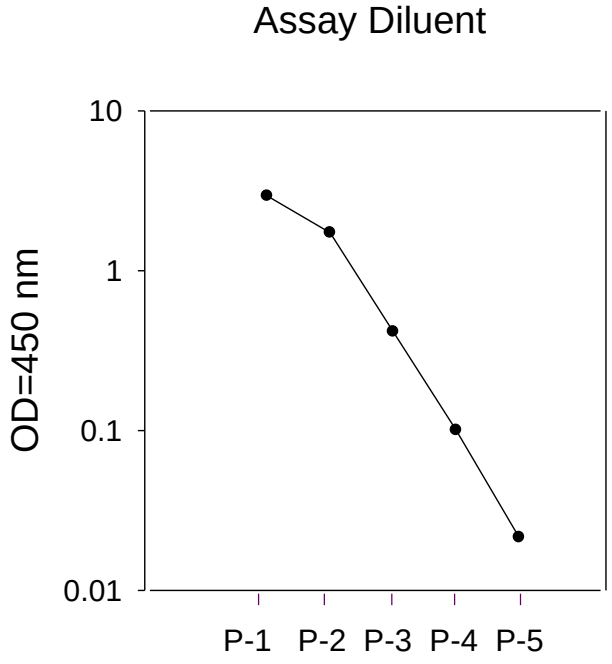
6. Add 50 µl Stop Solution to each well.
   Read at 450 nm immediately.

IX. TYPICAL DATA

ELISA data analysis: Average the duplicate readings for each sample or positive control then subtract the average blank optical density.

i. Positive Control

A431 cells were treated with recombinant human EGF at 37^{o}C for 20 min. Solubilize cells at 4 x 10^{7} cells/ml in Cell Lysate Buffer. Serial dilutions of lysates were analyzed in this ELISA. Please see step 3 of Part VI. Reagent Preparation for detail.

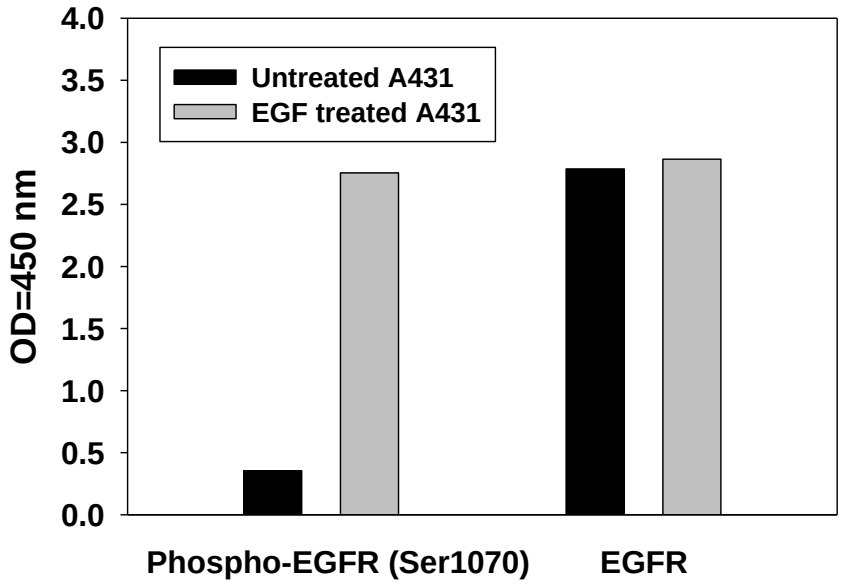


Positive control dilution series

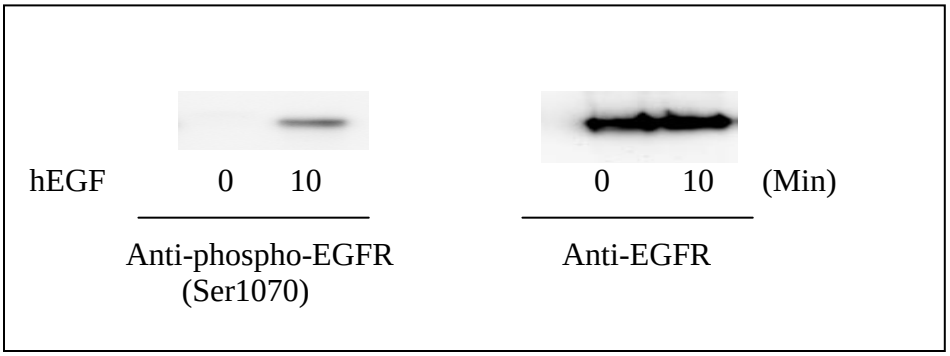
ii. Recombinant Human EGF Stimulation of A431 Cell Lines

A431 cells were treated or untreated with 100 ng/ml recombinant human EGF for 10 min. Cell lysates were analyzed using this phosphoELISA and Western Blot.

ELISA



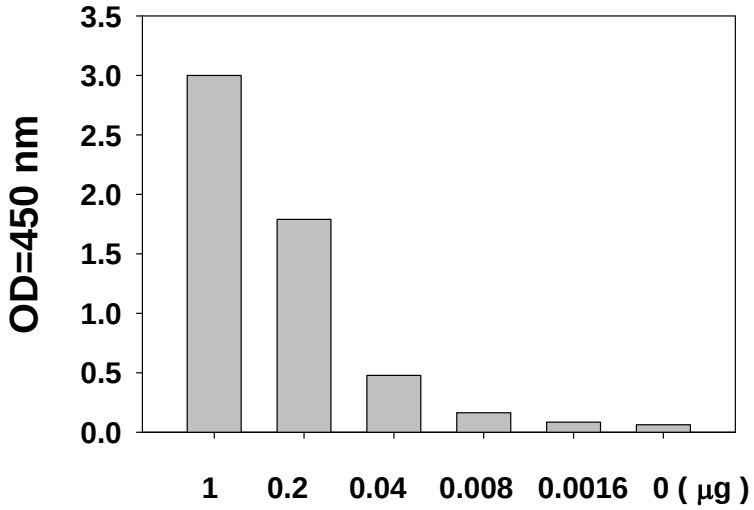
Western-Blot



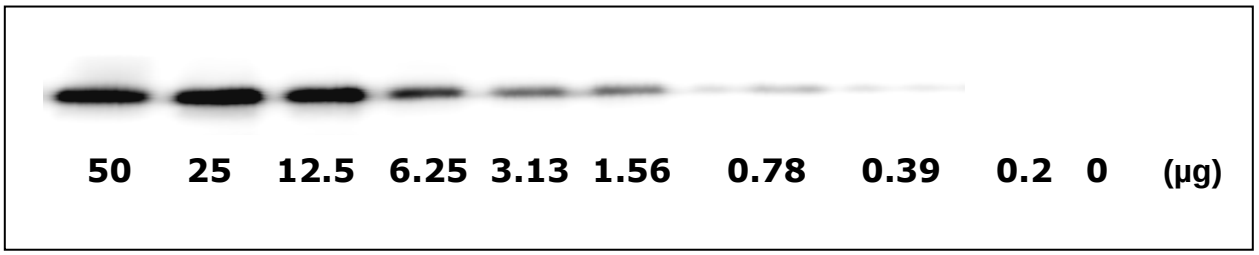
iii. SENSITIVITY

The A431 cells were treated with 100 ng/mL recombinant human EGF for 20 minutes to induce phosphorylation of EGF R. Serial dilutions of lysates were analyzed in this ELISA and by Western blot. Immunoblots were incubated with anti-phospho-EGFR (Ser 1070).

ELISA



Western-Blot



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XI. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Sample signals:		
a. Too low	a. Sample concentration is too low	a. Increasing sample concentration
b. Too high	b. Sample concentration is too high	b. Reducing sample concentration
2. Large CV	a. Inaccurate pipetting	a. Check pipettes
3. High background	a. Plate is insufficiently washed	a. Review the manual for proper washing. If using an automated plate washer, check that all ports are unobstructed.
	b. Contaminated wash buffer	b. Make fresh wash buffer
4. Positive Control: Low signal	a. Improper storage of the ELISA kit	a. Upon receipt, the kit should be stored at –20 ^{o}C. Store the positive control at -70^{o}C after reconstitution.
	b. Stop solution	b. Stop solution should be added to each well before measurement and read OD immediately.
	c. Improper primary or secondary antibody dilution	c. Ensure correct dilution

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Protein Array

ELISA

Cell-Based Phosphorylation ELISA

Tissue MicroArray

Protein: Cytokine, Chemokine, Adiplokine, Angiogenic factor, Virus, bacteria and infectious disease protein, hormone, Enzyme, other

Peptide

Antibody: Cytokine, Adipokine, Angiogenic factor, Signal transduction, Transcription factor, Receptor, Adhesion molecule, Virus, bacteria and other infectious agents, Secondary antibody, Tag antibody, Immunoglobulin, Hormone, Cell surface, Protease, other

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